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14. ABSTRACT

Ionizing radiation(IR)induces the activation of PI3K/Akt signaling pathway, which in turn regulates endothelial cell viability during treatment with radiotherapy. Inhibition of this pathway by receptor tyrosine kinase inhibitor (TKIs) enhances the cytotoxic effects of radiation in tumor vascular endothelium resulting in improved tumor control. There is significant evidence that multiple receptor tyrosinse kinases may be aberrantly activated in the prostate cancer cells. Therefore, we sought to study the effects of broad spectrum small molecule TKIs in the prostate tumor models. We demonstrate that inhibition of this pathway results in improved control of prostate tumor treated with IR via dual effect on both the tumor cells, and its microvasculature. Using innovative proteomic technology, we plan to identify the molecular profiles that are predictive of response to TKI and IR therapy. Our goal is for these results to provide insights in designing clinical studies aimed at taking these promising pipeline compounds to help treat patients with high risk prostate cancer.

15. SUBJECT TERMS
radiation oncology, proteomics, prostate cancer, molecular profiling, receptor tyrosine in kinase inhibitors

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Title: Molecular Profiling of Prostate Cancer To Determine Predictive Markers of Response & Susceptibility to Radiation and Receptor Tyrosine Kinase (TKI) Inhibitor Therapy.

Introduction:

Our laboratory has previously demonstrated that ionizing radiation (IR) induces the activation of PI3K/Akt, which in turn regulates endothelial cell viability during treatment with radiotherapy. Interestingly, inhibition of this pathway by receptor tyrosine kinase inhibitor (TKIs) enhances the cytotoxic effects of radiation in tumor vascular endothelium resulting in improved tumor control. In several prostate cancer cells, as in many cancer cells, there is constitutive activation of Akt. Furthermore, there is significant amount of evidence that multiple receptor tyrosinse kinases may be aberrantly activated in the prostate cancer cells. Therefore, we sought to study the effects of broad spectrum small molecule tyrosine kinase inhibitors in the prostate tumor models. We hypothesized that inhibition of this RTK/PI3K/AKT pathway will result in radiosensitization of prostate tumors via dual effect on both the tumor cells, and its microvasculature. Furthermore, by innovative approach of molecular profiling using state of the art proteomic technology (Matrix Assisted Laser Desorption Ionization or MALDI-imaging), we hypothesize that we can identify tumors with a molecular profile that are predictive of response to TKI and radiation therapy (RT). The techniques established in this study to determine the pharmacokinetics and molecular profile to TKI, can be applicable to other therapeutic agents. Our goal is for the results of these preclinical studies to provide mechanistic insights for the formulation of clinical studies aimed at taking these promising pipeline compounds to help treat patients with high risk prostate cancer.

Abbreviations:
EGFR = Epidermal Growth Factor Receptor
IR = Ionizing Radiation
IP = Intraperitoneal
MALDI = Matrix Assisted Laser Desorption Ionization
uM = microMolar
nM = nano Molar
PSA = Prostate Specific Antigen
PK = Pharmacokinetic
RT = Radiation Therapy
TKI = Tyrosine Kinase Inhibitor
VEGFR = Vascular Endothelial Growth Factor Receptor

Body

We have screened several compounds, and have decided to pursue AEE788 (Novartis) for our first set of studies for a number of reasons. AEE788 is a novel orally administered compound, currently in preclinical studies. AEE788 potently inhibits both EGFR and VEGFR in the nanomolar range[1]. It is effective in inhibiting tumor growth in a number of xenograft models, including prostate cancer DU145, and PC-3MM3 xenografts[1, 2]. This is likely due to the fact that EGFR is known to be expressed in prostate cancer cells, and has been demonstrated [3, 4] to play a role in tumor aggressiveness (Gleason Score, PSA), proliferation, angiogenesis, and migration [5]. Therefore, a dual inhibitor of EGFR and VEGFR should display significant anti-tumor effect by targeting both the EGFR expressing prostate tumor and its VEGFR expressing microvasculature.

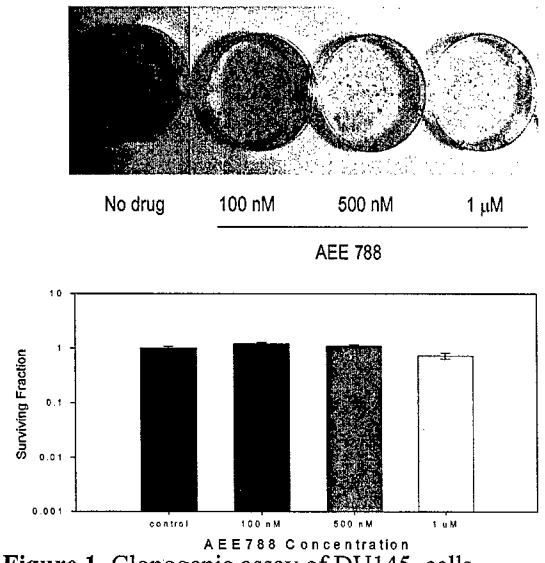

Figure 1. Clonogenic assay of DU145 cells treated with increasing dose of AEE788.

Experiment 1. AEE788 treatment of DU145 prostate cancer cells results in decreased cell proliferation. There have been reports of demonstrated resistance to EGFR inhibitor therapy in PTEN mutant cell lines due to chronic activation of PI3K/AKT pathway [6] Therefore, we initiated our studies using DU145, hormone resistant prostate cancer cells, which have EGF receptors and intact PTEN proteins[7, 8]. Clonogenic survival assay was performed by treating DU145 cells with increasing doses of AEE788. Interestingly, the surviving fraction was not affected by AEE788 at up to 1 uM doses, but there was a clear reduction in the size of the individual surviving colonies (fig. 1 upper panel). Therefore, AEE788 appeared to decrease cell proliferation of DU145 cells at as low as 100 nM dose, compared to vehicle treated counterparts. In fact, if harvested at an earlier time point, the data could have been mis-interpreted, as the treated cells may have had colonies < 50 cells. It was clear that longer incubations are necessary to accurately assess AEE788 effects on these cells. To confirm these findings, we performed a cell proliferation assay of DU145 cells. Exponentially growing DU145 cells were treated with increasing doses of AEE788 or vehicle control. Cells were counted every other day and the cell growth curve for each condition was plotted as demonstrated in figure 2. There was a dose dependent decrease in cell growth as expected, as early as at 100 nM concentration similar to our observation in the clonogenic cell proliferation of figure 1.

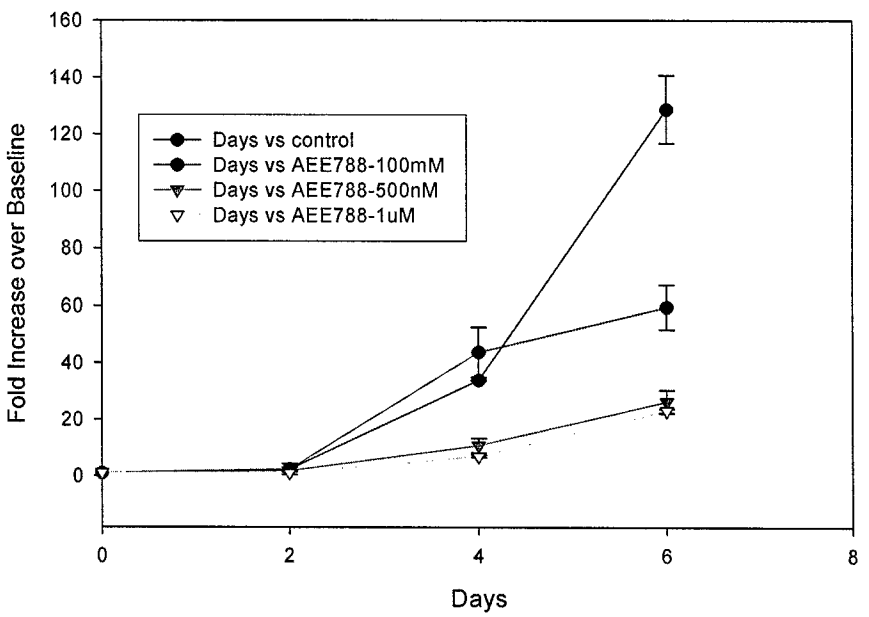

Figure 2. Cell growth curve of DU145 cells treated with increasing Doses of AEE788.

Experiment 2. AEE788 therapy leads to decreased proliferation of DU145 prostate cancer cells surviving radiation therapy. When the DU145 cells were treated with increasing doses of ionizing radiation, as expected, dose dependent decrease in surviving fraction (SF) was noted in these cells (fig 3a,c) without changes in individual colony size. Combination therapy of RT with AEE788 did not further reduce survival fraction of these cells, but once again significantly reduced the growth rate of colonies which survived the cytotoxic effects of radiation (2-6 Gy)(fig. 3b). These data suggest that the mechanism of decreased proliferation of AEE788 on the prostate tumor cells was independent of the mechanism of cytotoxic effect of ionizing radiation. This suggests that *in vivo,* inhibition of EGFR in the tumors may lead to delayed tumor growth in the cells that survive radiation therapy.

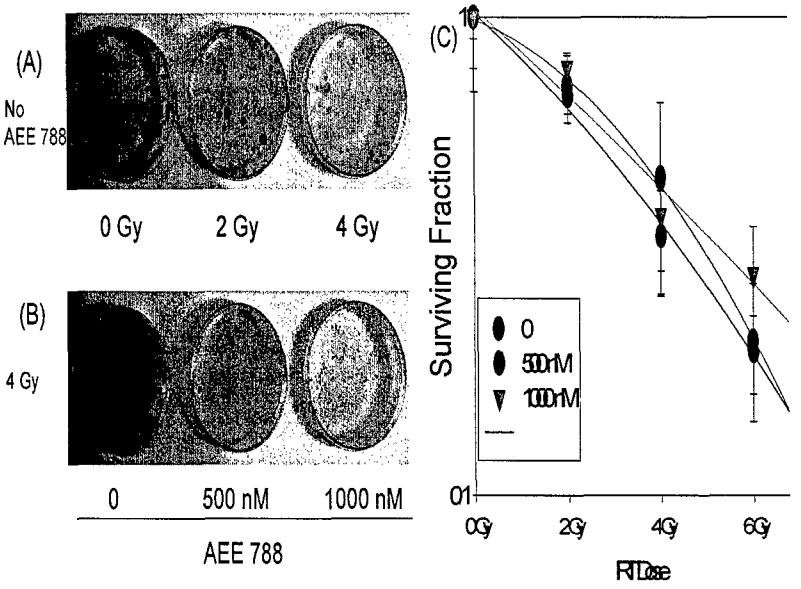

Figure 3. A)Representative clonogenic assay of DU145 cells (200 cells/plate) treated with RT. B) Representative clonogenic assay of DU145 cells treated with 4 Gy + AEE788 (500 cells/plate). C)Clonogenic survival curves of DU145 cells treated with RT and AEE788.

Experiment 3. Combination therapy of AEE788 and RT leads to increased prostate tumor growth delay *in vivo*. To determine the effects of AEE788 +/- RT *in vivo*, we performed a tumor volume growth delay experiment. DU145 prostate cancer cells were implanted on the hind limb of nude mice. When tumors reached a palpable size, the mice were then treated with suboptimal doses of AEE 788 (25 mg/kg), RT (3Gy), or RT +AEE788 (same doses) consecutively for seven days. Tumor volumes were measured for up to 40 days after initiation of therapy. There was a marked increase in tumor growth delay in the animals that were treated with AEE788 and RT (fig. 4) compared to each agents alone. Interestingly AEE788 even at suboptimal doses were effective at inducing tumor growth delay in this hormone independent, metastatic xenograft model.

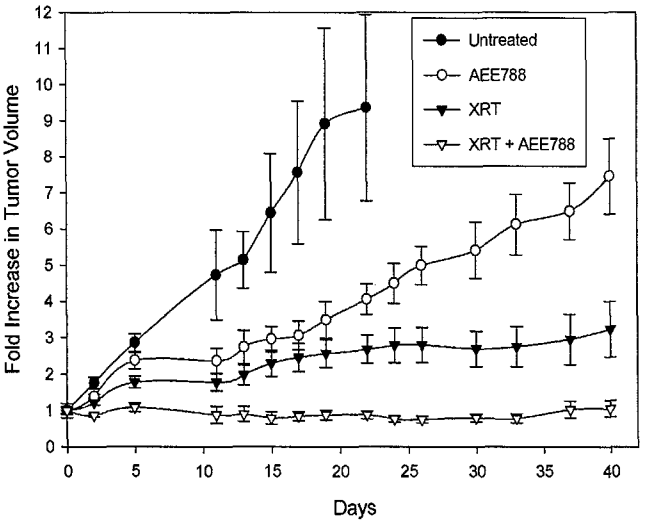

Figure 4. Tumor volume curves for nude mice bearing DU145 xenografts treated with AEE788, XRT (3Gy), XRT

Experiment 4. Combination therapy of AEE788 and RT leads to significant reduction in phospho-AKT levels 24 hours post therapy.

As AKT is a potential downstream target of EGFR in the prostate tumors, and since activation of AKT is often correlated with survival and cell proliferation, we performed immunohistochemistry studies on DU145 xenograft tumors harvested after 24 hrs from nude mice treated similarly as in experiment 3 above as indicated (figure 5) using an antibody specific for phospho-AKT. As seen in figure 5, vehicle treated tumors displayed a significant amount of phospho-AKT levels in this non-PTEN mutant prostate xenografts. In animals that were treated with AEE788 or AEE788+3Gy, there was a significant decrease in p-AKT level staining, while 3Gy alone had little effect (figure 5).

Experiment 5. Combination therapy of AEE788 and RT leads to significant reduction in tumor blood flow. The improved tumor growth delay noted in fig 4 could be due to the radiation independent anti-proliferative action of AEE788 on the prostate tumors, and/or due to effective destruction of the tumor vasculature due to combined inhibition of VEGFR and RT of the tumor endothelium. The same animals which were subjected to tumor volume measurement analysis in fig 4 were measured longitudinally for tumor blood flow using non-invasive Doppler Ultrasound as we have done previously [9] on days 0 and day 5 of therapy. As demonstrated in fig 6, animals that were treated with RT and AEE788 demonstrated statistically significant decrease in tumor blood flow compared to day 0 as assessed by percent change in power weighted pixel density (PWPD) measurements (p=.03). Animals treated with either vehicle, AEE788, or RT alone did not demonstrate significant reduction in tumor blood flow. This suggests that RTK/PI3K/AKT inhibition imposed by AEE788 in the tumor vasculature led to increased susceptibility to cytotoxic effects of radiation therapy as was seen with SU11248 in our laboratory's previous work [9]. These preliminary data suggest that AEE788 has at least two independent mechanism of improving prostate cancer therapy when combined with radiation: (1) tumor vasculature destruction via its VEGFR/PI3K/AKT inhibiting effects and (2) decreased proliferation of tumor cells surviving cytotoxic effects of radiation therapy.

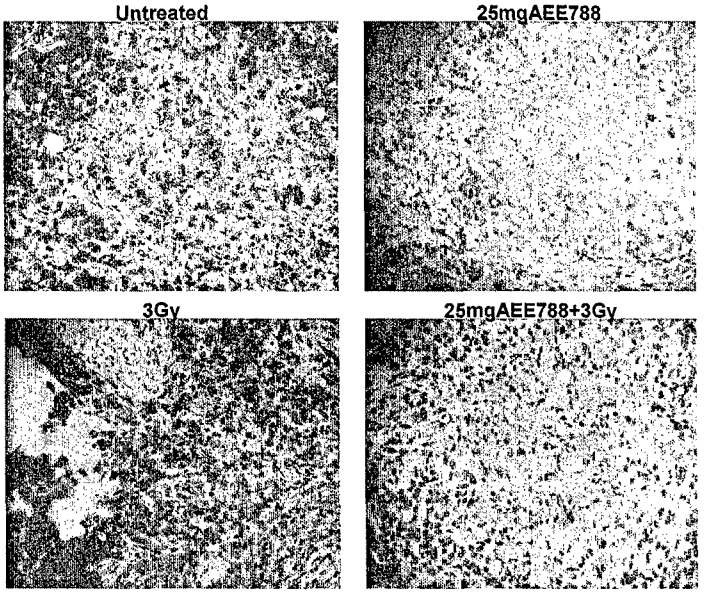

Figure 5. Immunohistochemistry of DU145 xenograft tumors harvested after treatment with indicated conditions for phospho-AKT levels

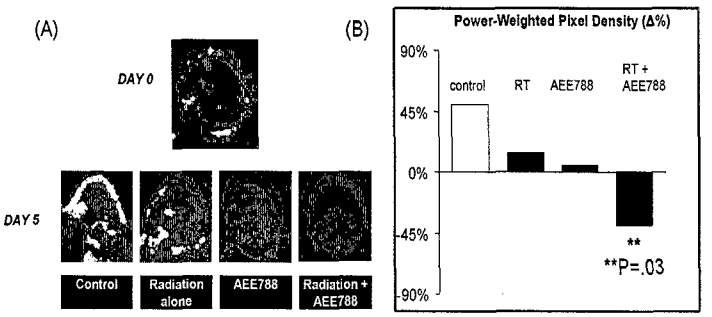

Figure 6. (A)Representative noninvasive color doppler sonography images from animals bearing DU145 tumor xenografts at day 0, and day 5 treated as indicated. (B) Graph demonstrates the change in power weighted pixel density (PWPD) at day 5 compared to day 0. ** = statistical significant.

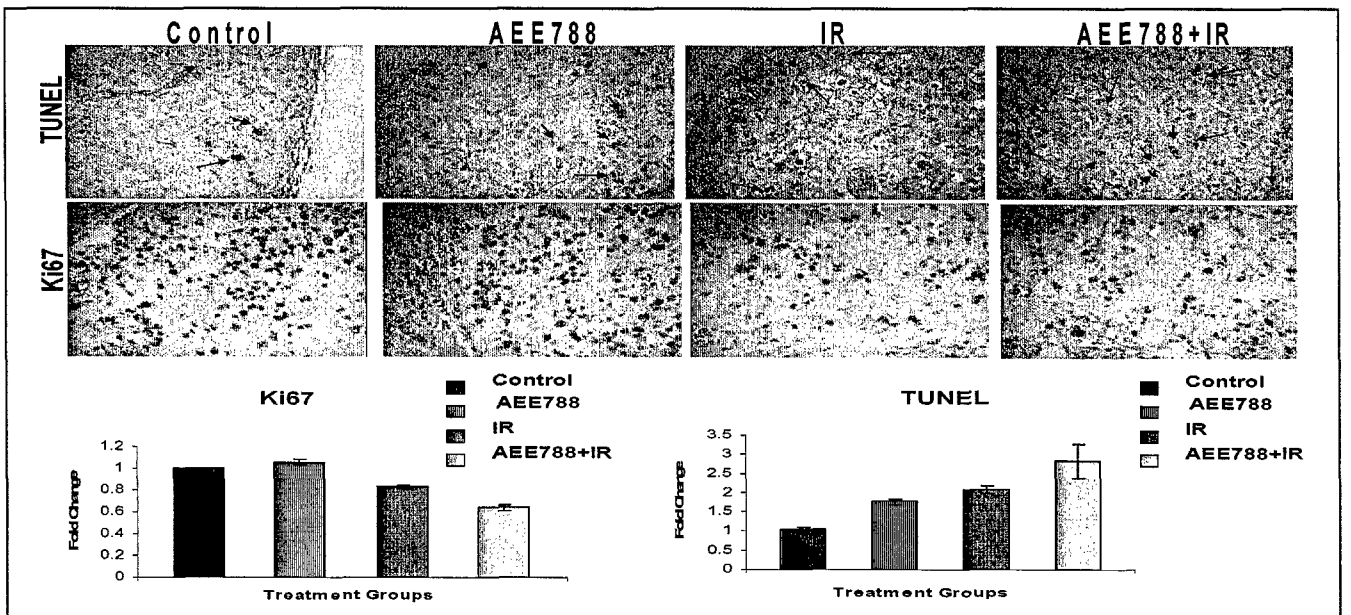

Figure 7. TUNEL and Ki-67 staining of DU145 tumors treated as indicated above.IR=ionizing radiation

Experiment 6. Pharamcodynamic analysis of prostate tumors from animals reated with AEE788 and/or RT demonstrates increased apoptosis, and decreased proliferation in the combination therapy group. To perform preclinical pharmacodynamics assessment of combination therapy on the tumors, we performed immunohistochemistry experiments for apoptosis (TUNEL staining) and proliferation (Ki-67 staining) in tumors harvested from animals that were treated with the indicated conditions (figure 7). As noted in the representative immunohistochemistry photos in the upper panels, with graphical representation in the lower panels, tumors that were treated with combination of AEE788 and RT led to highest level of apoptosis, and had the lower proliferative rate. This is consistent with our hypothesis that the dual inhibition of EGFR and VEGFR may lead to increased cytotoxicity and decreased proliferation of tumors undergoing radiation therapy. This data suggests that the differential profile of Ki-67 and TUNEL staining may prove to be good pharmacodynamics parameters to assess for response to AEE788 and RT therapy in the clinical setting from patient biopsy specimens.

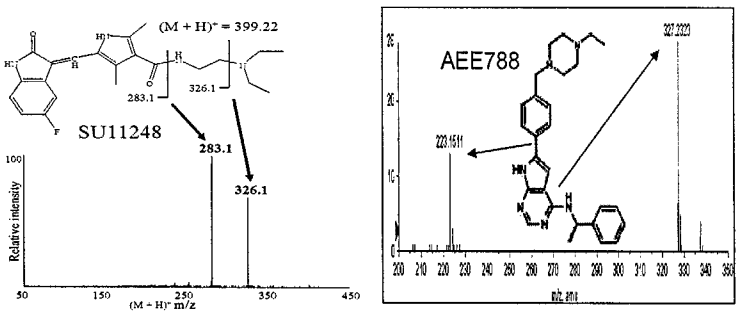

Figure 8. TKIs (SU11248 and AEE788) are ionizable, and detected by MALDI-MS. SU11248 is identified by two fragment ions of m/z ratio of 283.1 and 326.1, and AEE788 is identified by two fragment ions of m/z ratio of 223 and 327.

Experiment 7. TKIs such as AEE788, Gleevec, Tarceva, and SU11248 can be ionized and detected by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis. To use MALDI-MS technology for determining a drug's spatial biodistribution in tissue, we have to first determine that the drug is ionizable and detectable by MALDI-MS analysis. We and our collaborators have now confirmed that multiple TKIs (SU11248, AEE788, Gleevec, Tarceva) are detectable by MALDI analysis. Briefly, proteins or drugs of interest on a tissue on a MALDI plate is ionized (addition of positive charge) by a laser of set frequency which causes the proteins or drugs to desorb (detach) from the tissue or the plate. These proteins or compounds can then be separated by size and detected on a time of flight analyzer. SU11248 and AEE788 for example, when subjected to MALDI-MS, fragments at two specific sites of mass to charge (M/Z) ratio 283.1 and 326.1 (for SU11248), and 223 and 327 (for AEE788) as noted in fig 8.

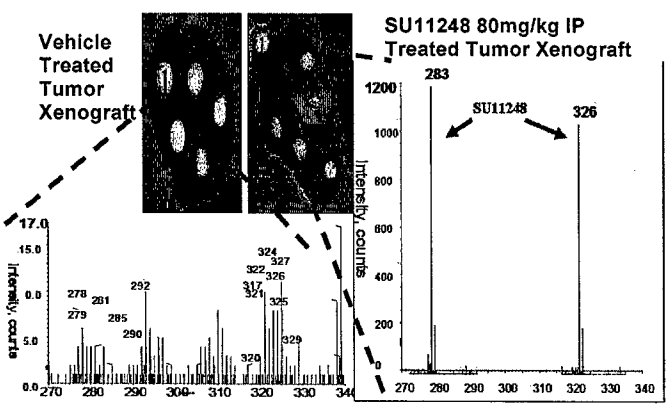

Figure 9. SU11248 can be detected directly from frozen tumor sections of treated animals by MALDI–TOF.

Experiment 8. TKIs administered intraperitoneally (i.p.) or orally (p.o.) to tumor bearing animals can be detected directly from the tumor tissues. Having determined that TKI compounds can be ionized and detected, we sought to determine whether we can detect the compounds directly from a frozen tissue section of a tumor from an animal that has been treated with TKIs. Animals bearing tumor xenografts on their hind limbs were treated with TKIs and sacrificed at various time points. Tumors were excised, frozen, and 12 uM sections were taken and placed on to a MALDI plate, matrix applied, and analyzed by MALDI-MS analyzer. Shown on fig 9 are representative tumors, one on the left representing a control animal treated with vehicle, and the tumor section on the right representing an animal treated with SU11248 at 80 mg/kg by IP injection. The circles seen on the tumors are matrix material which aids in the desorption and ionization process. We were able to detect the SU11248 peaks (M/Z 283 and 326), as demonstrated in fig 9, in animals treated with the drug, while the vehicle treated animals had an absence of the SU11248 peaks. Similar experiments were performed on prostate xenograft bearing animals to verify detectability of AEE788 in the prostate tumors (data not shown).

Experiment 9. Spatial biodistribution of TKIs can be assessed using MALDI-MS imaging. MALDI-MS-Imaging was performed on frozen DU145 prostate xenograft tumor tissue sections of mice treated with AEE788 at 80 mg/kg by p.o. administration. Tumors were imaged for AEE788 fragment of m/z 327 at 3-24 h post therapy. Shown in fig 10 is a tumor tissue imaged 6 h post therapy. The pattern of AEE788 compound detection, up to 24 h post therapy (data not shown) correlates with the published PK profile of AEE788[1].

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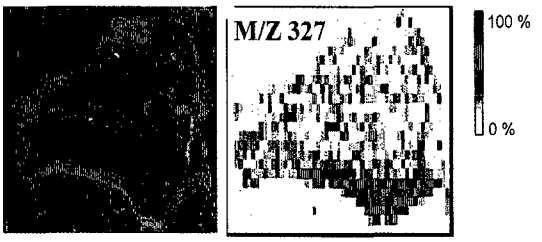

AEE788

Figure 10. AEE788 peaks can be imaged directly from frozen tumor tissue sections to determine the spatial biodistribution of the compounds.

Key Research Accomplishments

- Abstract accepted for ASTRO (American Society of Therapeutic Radiation Oncology) Annual Meeting (2005) for poster discussion session where PI (Dong Kim) is the last author (first author is the research assistant working under Dong Kim on this DOD funded project).
    - J.Huamani, K.J. Niermann, M.L. Reyzer, J. Alberts, C. Jones, R. Caprioli, D.E. Hallahan, **D.W. Kim**. Combination Therapy of Ionizing Radiation with AEE788, a Dual Receptor Tyrosine Kinase Inhibitor Targeting Epidermal Growth Factor Receptor (EGFR) and Vascular Endothelial Growth Factor Receptor (VEGFR) Leads To Improved Tumor Control. Poster Discussion, ASTRO 2005.

- Abstract accepted for oral presentation for Radiological Society of North America (RSNA 2005) Annual meeting; P.I. also received RSNA Trainee Research Prize.
    - **D.W. Kim,** M.L. Reyzer, J. Huamani, K. J. Niermann, R. Caprioli, D.E. Hallahan. Direct Analysis of Protein Markers of Therapeutic Response in Tumors Treated with Radiation and Receptor Tyrosine Kinase Inhibitors (TKI) by Imaging Mass Spectrometry. RSNA 2005.

- Manuscript in preparation
    - **D.W. Kim,** J.Huamani, K. Niermann, L. Geng, P. Traxler, D.E. Hallahan. Dual inhibition of EGFR and VEGFR in combination with ionizing radiation as a therapeutic strategy for prostate cancer. Manuscript in preparation.

- Technique for performing MALDI-imaging for receptor tyrosine kinase compounds including AEE788, SU11248 and Gleevec has been developed in collaboration with Vanderbilt Mass Spectrometry Resarch Center. Drug distribution analysis, as well as protein profiling of the tumors are now being performed using these established techniques.

Reportable Outcomes

1. Work supported by this grant is being presented as poster discussion at ASTRO 2005 national meeting (largest meeting for Radiation Oncologist).

2. Part of the work supported by this grant is being presented as an ORAL PRESENTATION at RSNA 2005 national meeting (largest meeting for Radiologist). Dong Kim (P.I.) also received a Trainee Research Prize to attend this meeting.

3. Work supported by this grant had led to significant preliminary data, which allowed Dong Kim to apply for the New Investigator Award from the DOD in 2005. Unfortunately, this grant was not funded, but received a favorable score (1.7).

4. P.I. Dong Kim is in his final year of training, and is in the process of applying for tenure track faculty positions. His application has been significantly strengthened by the training/experience supported by this grant.

Conclusions

- Spatial biodistribution of AEE788 was visualized in prostate tumor tissue specimens 2-24 hours following oral administration of this compound using MALDI imaging technology.
- Efficacy of tumor vasculature targeting by combination therapy (AEE788+IR) was evidenced by reduction in tumor blood flow demonstrated by non-invasive imaging modalities (doppler sonography and microbubble constrast enhanced sonography
- Combination therapy with AEE788 and IR led to significant tumor growth delay in human prostate tumor xenografts
- Mechanisms of growth reduction in the combination therapy group, included decreased tumor proliferation rate, vasculature destruction, and increased apoptosis.
- P.I. (Dong Kim) has benefited tremendously from the experience/training supported by this grant thus far. Plan is for continued studies as proposed in the grant. Further plans include preparation of 1-2 manuscripts based on the results from these studies in the coming year. Finally, P.I. is currently applying for faculty positions to begin at the end of this training period (2006), and is planning to apply for grants to continue to support this research project.

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